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10/29/03 Date
Mark B. Wilson

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Conrad

Serial No.: 09/854,412

Filed: May 11, 2001

For: HIGH EFFICIENCY mRNA ISOLATION METHODS AND COMPOSITIONS

Group Art Unit: 1636

Examiner: Katcheves, Konstantina

Atty. Dkt. No.: AMBI:073US

CORRECTED DECLARATION OF RICHARD C. CONRAD, PH.D, UNDER 37 C.F.R. §1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Richard C. Conrad, Ph.D., declare the following:

1. I am an inventor of the above-referenced patent application. I am a Senior Scientist at Ambion, Inc. and have worked there for three years (since March, 2000). I have a Ph.D. in Molecular Biology, which I received in 1987 from The University of Wisconsin at Madison. I was a postdoctoral fellow at Indiana University for nine years and at Eli Lily and Company for two and a half years, as well as Facility Manager at Indiana University for two years. I have worked in the field of molecular biology, including nucleic isolation

techniques for approximately twenty-five years. My *curriculum vitae* is attached as Exhibit 1.

2. I understand that the claims in this application have been rejected as not novel or obvious over U.S. Patent No. 5,759,777 issued in the name of Kearney *et al.* ("Kearney patent").

3. I have reviewed the Kearney patent and believe it does not disclose or teach my invention.

4. My invention is based on my discovery that some problems with mRNA isolation stems from rRNA carryover that is based not on rRNA interactions with the targeting molecule, such as oligo-dT, but on rRNA interactions with mRNA. *See* specification at page 4, lines 25-28; Examples 1 and 2.

5. The use of TEAC and TMAC minimizes differences in bond strength between A:T and G:C basepairs, as G:C basepairing is known to be stronger than A:T basepairing. Isolation of mRNA based on A:T basepairing is affected in the presence of TEAC or TMAC. Stretches of A:T basepairing between mRNA and a poly(T) or poly(U) nucleic acid can be positively exploited at the expense of G:C and A:T basepairing between mRNA and rRNA to reduce the carryover of rRNA. *See* specification at page 4, line 28 to page 5, line 7. Furthermore, I believe the TEAC and TMAC reduce basepairing between the rRNA and mRNA, as well as rRNA and a poly(T) or poly(U) nucleic acid that might be employed to hybridize with the mRNA.

6. Based on my knowledge of the field, I believe that if one did not know or appreciate that rRNA carryover as a contaminant in a mRNA sample can be attributed to hybridization between rRNA and mRNA or between rRNA and a poly(T) or poly(U) nucleic acid, then

that person would not consider the use of TEAC or TMAC in an mRNA isolation procedure.

7. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Oct 29, 2003

Date

Richard C. Conrad

Richard C. Conrad, Ph.D.

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Specificity of Oligo (dT)-Cellulose Chromatography in the Isolation of Polyadenylated RNA

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Nonspecific types of binding occur when oligo(dT)-cellulose is used to analyze or prepare poly(A)RNA. First, nonpolyadenylated nucleic acids bind and are eluted under conditions used to elute poly(A)RNA. Second, "tight" nonspecific binding occurs in which poly(A)RNA fails to elute under conditions which dissociate A-T bonds. Hydrolysis is required to remove tightly bound RNA. Oligo(dT)-cellulose has a low capacity for both these types of binding, and can be readily preempted with a heterologous RNA e.g., bacterial. Third, indirect nonspecific binding can also occur. rRNA aggregates with poly(A)RNA and thus can bind indirectly to oligo(dT)-cellulose. After these aggregates are disrupted by treatment with DMSO and heat, poly(A)mRNA free of rRNA can be isolated. Efficient recovery of poly(A)hnRNA from total nuclear RNA is accomplished using oligo(dT)-cellulose if the RNA is first subjected to conditions which disrupt aggregates and reduce secondary structure. Ninety-five to ninety-eight per cent of the purified poly(A)hnRNA and poly(A)mRNA rebinds to oligo(dT)-cellulose.

Oligodeoxythymidylic acid-cellulose (oligo (dT)-cellulose), used by Aviv and Leder (1) for the isolation of translatable poly(A)mRNA, has been used extensively for the isolation of poly(A)RNA from a variety of sources (2-6). We have used oligo(dT-cellulose columns both preparatively and analytically and have encountered certain problems which led us to evaluate the specificity and efficiency of A-T hybridization chromatography. Since oligo(dT)-cellulose chromatography provides a rapid and simple method for the isolation of poly(A)RNA from various bulk RNA preparations (total cellular, nuclear, polysomal, etc.), attention to details which improve specificity and increase recovery is warranted.

We report here experiments which demonstrate the specificity and efficiency of oligo(dT)-cellulose chromatography, and we present methodology which is effective in preventing nonspecific and indirect binding of nonpolyadenylated RNA species.

METHODS

Nucleic Acids

Tritium labeled and unlabeled adenylic and uridylic acid homopolymers were from Miles Laboratories. RNA was purified from whole cells,

polysomes, or nuclei of adult mouse brains. Two methods of extraction were employed depending on which subcellular organelle was being extracted. For the extraction of nuclear and whole cell RNA, the pH 5.2 hot phenol technique of Edmonds and Caramela (7) was used. This technique conserves poly(A)RNA and separates DNA from RNA by permitting selective retention of DNA in the phenol phase. This was essential since high molecular weight DNA clogs oligo(dT)–cellulose during poly(A)RNA isolation. We found that "RNAase free" DNAase (Worthington DPFF grade) could not be used prior to oligo(dT)–cellulose chromatography because cleavage of the RNA occurred. The phenol-chloroform method of Perry *et al.* (8) was used to extract RNA from polysomes.

Ribosomal RNA (^{14}C) was prepared from mouse L cells by the naphthalene disulfonate method of Kirby (9) and purified further by sucrose gradient centrifugation. *E. coli* RNA was prepared by the method of Edmonds and Caramella (7) and treated with DNAase (1.5 hr, 37°C, 250 μg/ml Worthington DPFF DNAase). An additional phenol extraction was performed to remove the DNAase. ^{3}H-labeled, single-strand, unique sequence DNA and native sheared DNA were prepared from mouse L cells by the method of Hahn and Laird (10).

Glassware and buffers used in the preparation and fractionation of nucleic acids were sterilized.

Preparation of Nuclei and Polysomes

Nuclei were prepared at 0–4°C by homogenizing 50 whole mouse brains with a Teflon–glass motorized homogenizer in 8 vol of buffer (0.25% Triton X-100, 0.32 M sucrose (RNAase free), 0.001 M KCl, 0.001 M $MgCl_2$, 0.01 M Na acetate, pH 6.0). The homogenate was passed through two layers of sterile cheesecloth and centrifuged at 2,500g av., 10 min, 0°C in a swinging bucket rotor. The supernatant was discarded and the nuclear pellet was resuspended in the same buffer, lacking Triton X-100, with five strokes of a Dounce homogenizer (A pestle). After pelleting the nuclei, they were again washed by resuspension and pelleting. The crude nuclear pellets were suspended in 190 ml of 2.2 M sucrose, 0.001 M KCl, 0.001 M $MgCl_2$, 0.01 M Na acetate pH 6.0, using the Dounce homogenizer, and layered over 3 ml pads of the same buffered sucrose in Beckman SW 27 centrifuge tubes. The interface was gently mixed and the tubes were centrifuged at 110,000g av., 75 min, 0°C. The nuclei which pelleted through the sucrose pad were then washed with 0.1 M Na acetate, pH 5.2 and RNA was extracted.

Polysomes were prepared at 0–4°C by a modification of the technique of Lee and Brawerman (11). Mouse brains were homogenized by five strokes of a Dounce homogenizer (A pestle) in 20 vol of buffer (0.1% Triton X-100, 5% w/v sucrose, 0.25 M KCl, 0.01 M $MgCl_2$, 0.05 M tris–HCl, pH 7.4 and 100 μg bentonite/g mouse brain). The homogenate was centrifuged, first at 3000g av., 10 min, 0°C and then at 17,000g av., 10 min, 0°C, (12). The resulting postmitochondrial supernatant was layered over a 10 ml pad of 20% w/v sucrose containing 0.25 M KCl, 0.01 M $MgCl_2$, 0.05 M tris–HCl, pH 7.4. After mixing the interface, the preparation was centrifuged at 110,000g av., 85 min, 0°C in a SW 27 rotor. The resulting polysomal pellet was extracted as stated in "Nucleic Acids."

Oligo(dT)–Cellulose

Cellulose containing covalently bound oligodeoxythymidylic acid residues (Length = 10–12 bases) was from Collaborative Research, Inc., Waltham, Massachusetts. Three grades (T2, T3, T3I) were tested. The T2 grade had the lowest binding capacity and the highest background "noise" (poly(A) – RNA binding). The T3 grade had two to five times greater binding capacity and less nonspecific binding than the T2 grade. Currently, Collaborative Research prepares T3 grade oligo(dT)–cellulose using Whatman CF 11-cellulose washed by the method of Alberts and Herrick (13). We refer to this improved grade as T3I (improved) oligo(dT)–cellulose while Collaborative Research refers to it as T3.

Oligo(dT)–Cellulose Chromatography

The binding and elution method of Aviv and Leder (1) was used except that NaCl was substituted for KCl so that sodium dodecyl sulfate (SDS) could be included to inhibit any RNAase. Ethylenediaminetetracetic acid disodium salt (EDTA) (1.0 mM) was also added to minimize divalent cation catalyzed cleavages. The figure legends indicate the elution procedure. Polysomal RNA was first heat-treated (65°C, 3 min) prior to column application. Nuclear and whole cell RNA were heat-treated in the presence of 80% v/v DMSO prior to chromatography on oligo(dT)–cellulose (see below). The buffers used were 0.5 M NaCl, 0.001 M EDTA, 0.01 M tris–HCl, pH 7.5; 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris–HCl, pH 7.5; and 0.01 M tris–HCl, 0.001 M EDTA, pH 7.5.

Treatment with Heat and DMSO

Aggregated RNA was dissociated by treatment with dimethylsulfoxide (DMSO) and heat prior to fractionation on oligo(dT)–cellulose or sucrose gradient centrifugation. RNA was dissolved in 1 vol of 0.01 M tris–HCl buffer, pH 7.5 to which 9 vol of DMSO (Mallinckrodt) was added. This was immediately followed by the addition of 1 vol of buffered 1 M LiCl (1 M LiCl, 0.05 M EDTA, 2.0% w/v SDS, 0.01 M tris–HCl, pH 6.5). If the RNA was to be fractionated over oligo(dT)–

cellulose, it was first held at 55°C for 5 min and then diluted 10 times with 0.5 M NaCl buffer at room temperature. If the RNA was to be sedimented through DMSO–sucrose gradients, it was heated to 37°C for 5 min and then diluted with 1 vol of buffered 0.1 M LiCl prior to application on the gradient.

Density Gradients

Since some RNA molecules readily aggregate, DMSO–sucrose gradients were routinely used. DMSO-treated RNA was layered over 5–20% w/v sucrose gradients containing 50% v/v DMSO, 0.1 M LiCl, 0.005 M EDTA, 0.2% w/v SDS, 0.01 M tris–HCl, pH 6.5. Details on these gradients will be published elsewhere. Sucrose gradients without DMSO (5–20% w/v sucrose, 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris–HCl, pH 7.4) were also used where indicated.

Poly(U) Hybridization

Percentage of poly(A) content was measured by hybridizing [^{3}H] poly(U) (Miles) to poly(A) tracts. [^{3}H]poly(U) (0.5 μg) (>50,000 cpm) was added to 3 μg samples of RNA. Samples were incubated at 65°C for 3 min and then at 37° for 3 hr in 0.5 ml of 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris–HCl, pH 7.5. Excess poly(U) and nonhybridized RNA were digested by the addition of 0.3 μg/ml RNAase A, (Worthington-RAF grade), 20 min, 28°C. Carrier RNA (35 μg/ml) was added and the sample precipitated with 5% w/v trichloracetic acid (TCA). After 20 min on ice, the solution was slowly filtered through 0.45 μm "Millipore" filters. Filters were washed six times with 1.0 ml applications of 5% TCA. After drying the filters, radioactivity was measured in toluene-Omnifluor cocktail (New England Nuclear). RNAse resistant poly(U) was used as a measure of the amount of poly(A) present in the RNA sample Using poly(A) homopolymer in various ratios with [^{3}H]poly(U), we estimated that about 7% of the poly(U) hybridized might be in triplex form in the assays we performed. Correction for this was made in estimating poly(A) content of poly(A)RNA. Background "noise" was determined by substituting *E. coli* RNA in the poly(U) assay. Noise estimated by this control was <4% of the observation. Hybridization of a known quantity of poly(A) homopolymer (Miles) to [^{3}H]poly(U) was used as a reaction standard.

RESULTS

Specificity of Oligo(dT)–Cellulose for Poly(A)

As Table 1 shows, oligo(dT)–cellulose (T3 grade) is highly specific for poly(A). Poly(A) homopolymer binds, but poly(U), sheared single strand DNA, double strand DNA and 4S *E. coli* RNA, all of which lack poly(A) do not. In comparison, poly(A) RNA isolated from polysomes which contains an average of 7.7% poly(A), binds virtually 100%.

TABLE 1

SPECIFICITY OF OLIGO (dT)–CELLULOSE (GRADE T3) FOR POLY(A) CONTAINING MOLECULES[a]

Nucleic acid	Bound (%)
poly(A) homopolymer	97
poly(U) homopolymer	1
poly(U)-poly(A) triplex	1
Sheared single strand DNA	0.1
Sheared double strand DNA	0.3
4S *E. coli* RNA	0.2
poly(A)mRNA	98.7

[a] ^{3}H-labeled nucleic acids were applied to preempted (see next section and Table 3; columns made from 0.25 g of oligo(dT)–cellulose. In all cases <1.0 μg (≈5000–15,000 cpm) of nucleic acid was applied.

Low Capacity Nonspecific Binding Sites

Despite the specificity for poly(A), two types of direct nonspecific binding occur One type of "site" binds poly(A) — nucleic acids which are eluted by the addition of 0.01 M tris–HCl or H_2O. Second, tight binding occurs in which hydrolysis is required to remove bound nucleic acids.

The extent of binding to the nonspecific 0.01 M tris–HCl elutable "sites" is related to the quality of the oligo(dT)–cellulose. Table 2 shows the results of testing three grades of oligo(dT)–cellulose. *E. coli* RNA bound more extensively to T2 and T3 grades of oligo(dT)–cellulose than to T3I (T3 improved). The main difference among these grades, aside from

TABLE 2

BINDING OF *E. coli* RNA TO VARIOUS GRADES OF OLIGO(dT)–CELLULOSE[a]

Grade	*E. coli* RNA bound/g oligo (dT)–cellulose (μg)
T2	4.5–5.0
T3	2.0–2.5
T3I	0.5–0.7

[a] Total cellular *E. coli* RNA (100 μg) was passed three times through 1 g columns of oligo(dT)–cellulose. Columns were washed to a background optical density with 0.5 M NaCl RNA eluted with 0.01 M tris–HCl constituted the bound fraction.

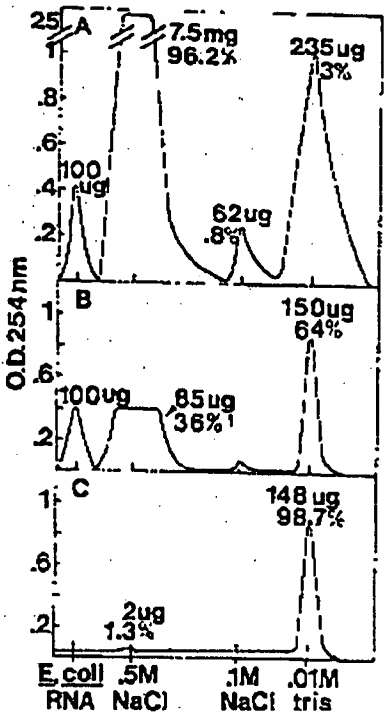

FIG. 1. Purification of poly(A)mRNA and prevention of indirect binding of rRNA. (A) RNA extracted from mouse brain polysomes was dissolved in 4.0 ml buffered 0.5 M NaCl and held at 65°C for 2 min. After cooling to room temperature, the RNA was passed three times through a 0.75 g T31 oligo(dT)–cellulose column. Optical density was monitored at 254 nm during final application, washing (0.5 M NaCl), and elution (0.1 M NaCl and 0.01 M tris–HCl). *E. coli* RNA was first washed through the column a single time to preempt nonspecific binding sites. Solvents used to elute fractions are given on the abscissa. (B) The RNA in the 0.01 M tris–HCl eluted fraction (poly(A)mRNA) was precipitated with ethanol, pelleted and dissolved in 20 μl 0.01 M tris–HCl, 180 μl DMSO and 20 μl buffered 1.0 M LiCl were then added. The solution was heated to 55°C for 5 min and then diluted 10-fold with buffered 0.5 M NaCl. Chromatography as in (A) was repeated. (C) Poly(A)mRNA (0.01 M tris–HCl fraction) from (B) was made 0.5 M with respect to NaCl and rechromatographed without preemption of the column.

Figure 1 shows the optical density scans obtained from the chromatography of total polysomal RNA on oligo(dT)–cellulose. The column was first preempted with *E. coli* RNA. The bulk of the polysomal RNA failed to bind since polysomal RNA is mostly rRNA. Upon lowering the salt to 0.1 M a small amount of RNA was eluted. Washing with 0.01 M tris–HCl removed the bound or "poly(A)RNA" fraction. Figure 1b shows the rebinding of the 0.01 M tris fraction shown in Fig. 1A. Rebinding was performed immediately after a disaggregation step in which

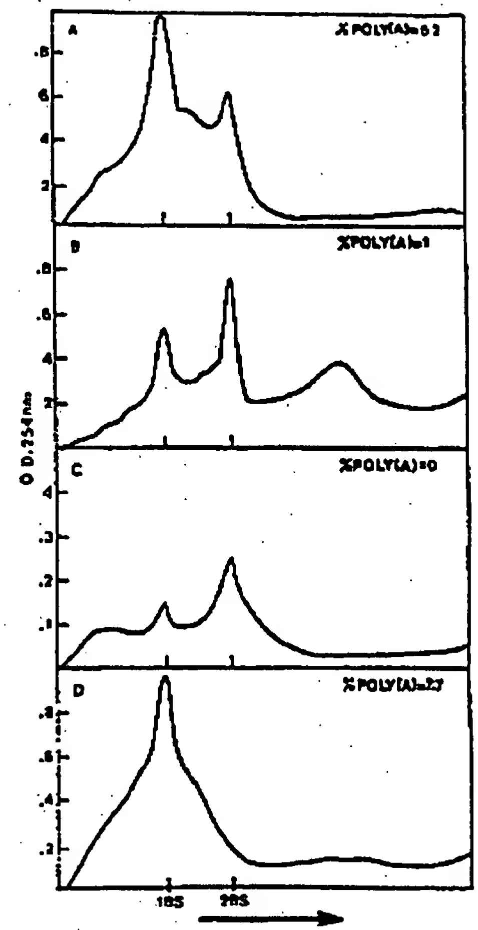

FIG. 2. Sedimentation of polysomal RNA Recovered at various steps during purification of poly(A)mRNA. (A) First step purified poly(A)mRNA (0.01 M tris–HCl fraction Fig. 1A) was dissolved in 10 μl 0.01 M tris–HCl, 90 μl DMSO and 10 μl of a solution containing 1.0 M LiCl, 0.05 M Na$_2$ EDTA, 2% SDS, and 0.1 M tris–HCl (pH 6.5) was then added. After heating to 37°C for 5 min, an equal volume of buffered 0.1 M LiCl was added. The solution was then layered over a 5–20% w/v sucrose gradient containing 50% v/v DMSO and buffered 0.1 M LiCl. Gradients were centrifuged in a SW41 rotor (Beckman) at 35,000 rpm for 14 hr at 20°C. Gradients were fractionated and monitored with an ISCO fractionator. (B) The 0.1 M NaCl eluted fraction seen in Fig. 1A was treated as described in (A). (C) The 0.5 M NaCl fraction shown in Fig. 1B was treated as described in *Fig.* 2A. (D) The 0.01 M tris–HCl fraction, Fig. 1B, was treated as described in Fig. 2A. The poly(A) content of the RNA fractions was measured by hybridization with ^{3}H-labeled poly(U) as described in procedures.

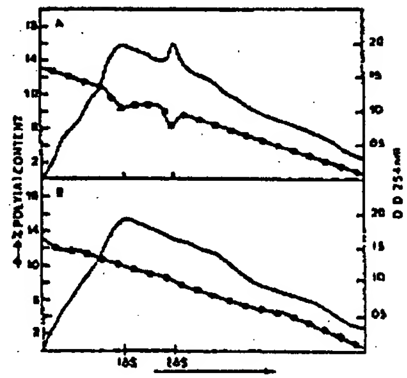

FIG. 3. Relationship of sedimentation rate to poly(A) content of first and second step purified total cellular poly(A) RNA. (A) First step purified poly(A), as shown in Fig. 1A, 0.01 M tris–HCl fraction, was sedimented in 5–20% w/v aqueous sucrose gradients for 5 hr, 0°C at 38,000 rpm in an SW41 rotor. Fractions were collected and the optical density of each determined. Poly(A) content of the RNA in each fraction was determined by the [^{3}H]poly(U) assay. (B) Second step bound poly(A)RNA as shown in Fig. 1B was sedimented as given for Fig. 2. Poly(A) content of fractions from the gradient was determined as stated in Fig. 3A.

and buffered 0.1 M LiCl. The preparation was then diluted to lower the concentration of DMSO to 10% and applied to the column. Figure 1B shows that 36% of the RNA failed to rebind. The bound fraction in Fig. 1B was then reapplied to oligo(dT)–cellulose, as shown in Fig. 1C, and 98.7% binding occurred.

Analysis of the poly(A) content and sedimentation rates of bound and unbound fractions from these various stages of purification on oligo(dT)–cellulose is shown in Fig. 2. Shown in Fig. 2A is the sedimentation of the 0.01 M tris–HCl eluted fraction from Fig. 1A. 18S and 28S peaks were observed in the profile of this first step purified poly(A)mRNA.

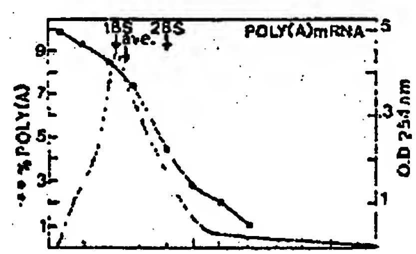

FIG. 4. Poly(A) content in relation to sedimentation rate of poly(A)mRNA. Twice bound poly(A)mRNA was sedimented in a DMSO–sucrose gradient as in Fig. 2. The gradient was fractionated and the RNA from each fraction was bound to oligo(dT)–cellulose to remove DMSO. The optical density of each fraction was measured at 260 nm and the poly(A) content determined by hybridization with [^{3}H]poly(U). Optical density scan which

In Fig. 2C, the sedimentation of the unbound (0.5 M NaCl) fraction shown in Fig. 1B is presented. This fraction was comprised largely of 18 and 28S RNA and had no measurable poly(A) content. As demonstrated in Fig. 2D, the second-step purified poly(A)mRNA fraction shown in Fig. 1B sedimented as a broad band with a median of about 20S.

In another experiment, the elimination of rRNA is demonstrated another way. Figure 3 shows the sedimentation of two preparations of total cellular poly(A)RNA. Figure 3A is the profile of poly(A)RNA prepared by a single binding to oligo(dT)–cellulose without a DMSO disaggregation step. The percentage of poly(A) content was measured on fractions from the gradient by hybridization with ^{3}H-labeled poly(U). Notice that at 18 and 28S "dips" in the poly(A) content appear. This is expected since rRNA lacks poly(A). Figure 3B shows that these "dips" are eliminated when total cellular poly(A)RNA is subjected to the DMSO disaggregation step and again bound to oligo(dT)–cellulose. Likewise, when twice bound poly(A)mRNA is analyzed for poly(A) content relative to sedimentation, as shown in Fig. 4, no decreases were noted at 18 and 28S. These observations indicate that *most* of the rRNA is eliminated by rebinding after the DMSO disaggregation step.

It has been our experience that the disaggregation step should be performed after the initial chromatography of total polysomal RNA. As Fig. 1A demonstrates, the vast bulk of the rRNA is eliminated during this passage. If disaggregation is performed when rRNA is present in high concentration, there is a tendency for reaggregation to occur after the DMSO is diluted.

The bound fraction shown in Fig. 1A which was eluted with 0.1 M NaCl, contained about 1% poly(A) and 15% of this RNA rebound to oligo(dT)–cellulose following disaggregation. The sedimentation profile shown in Fig. 2B indicates that this fraction is mostly 18 and 28S RNA but a third rapidly sedimenting heterogeneous component was observed. About 10% of the rapidly sedimenting component rebinds to oligo(dT)–cellulose and hybridizes with poly(U). Whether this poly(A)RNA exists as an aggregate with poly(A)$^-$RNA or is actually high molecular weight poly(A)RNA is not known. We have not analyzed the 0.1 M fraction further, but it is clear that some poly(A)RNA might be lost if samples are applied in 0.1 M NaCl, and the loss might be selective. Therefore, we recommend that RNA be applied to oligo(dT)–cellulose in the presence of 0.5 M NaCl.

Demonstration of the Aggregation of rRNA with poly(A)RNA

Evidence for the aggregation of rRNA with purified poly(A)RNA is presented in Table 5. Labeled 18S and 28S rRNA did not bind to *E. coli* RNA preempted oligo(dT)–cellulose. Labeled rRNA, after incubation with poly(A)$^-$polysomal RNA, also failed to bind. However, 44% of a [illegible] 18S [illegible] 28S rRNA bound after incubation (10 min, 0.5 M

TABLE 5

BINDING OF rRNA TO OLIGO(dT)-CELLULOSE IN THE PRESENCE OF POLY(A)RNA

RNA	Percentage eluted		
	0.5 M NaCl	0.1 M NaCl	0.01 M tris-HCl
18S	99.7	—	0.3
28S	99.8	—	0.2
18S + 2 mg poly(A)⁻RNA	99.9	0	0.05
28S + 2 mg poly(A)⁻RNA	99.9	0.07	0.03
18S and 28S + 187 μg poly(A)RNA	56.2	20.9	ᵃ22.9
Disaggregated 0.01 tris-HCl fraction	97.5	1.5	2

Note: 0.5 g of T3I oligo(dT)–cellulose was preempted with *E. coli* RNA and 7–20 μg [^{14}C]rRNA (specific activity = 650 cpm/μg) was applied in 0.5 M NaCl. When [^{14}C]rRNA was mixed with either poly(A)⁻ polysomal RNA or total cellular poly(A)RNA the incubation conditions were 20°C, 0.5 M NaCl for 10 min prior to application. The rRNA which was eluted with 0.01 M tris-HCl after incubation with poly(A)RNA (ᵃ22.9%) was reapplied to the column after heating in the presence of 80% v/v DMSO (disaggregated 0.01 tris-HCl fraction).

NaCl), with poly(A)RNA. This experiment demonstrates the strong tendency of rRNA to "bind" indirectly to oligo(dT)–cellulose as an aggregate with poly(A)RNA. Disaggregation with DMSO essentially eliminated the indirect binding of rRNA. This is shown in the last item of Table 5 in which rRNA eluted by 0.01 M tris was disaggregated from poly(A)RNA by heating in DMSO and reapplied to the column. Despite the presence of a relatively large amount of poly(A)RNA (about 180 μg), binding of rRNA was nearly eliminated.

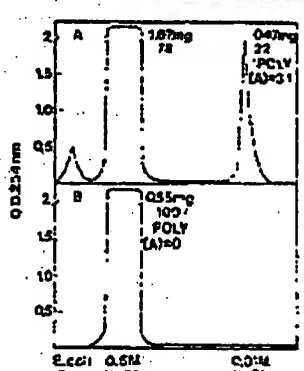

FIG. 5. Binding of poly(A)hnRNA to oligo(dT)–cellulose. (A) Total nuclear RNA from mouse brain was applied to a column of oligo(dT)–cellulose following disaggregation in DMSO as described for Fig. 1B. The column was washed and eluted as indicated. (B) The unbound fraction (0.5 M NaCl) from Fig. 4 was treated for 3 sec with 0.2 N NaOH at room temperature (5), neutralized and applied to oligo(dT)–cellulose. As shown, no additional RNA bound.

Recovery of poly(A)hnRNA

Concern over whether efficient recovery of poly(A)hnRNA is obtained has been voiced by several investigators. For example, Derman and Darnell (5) stated that about 50% of the >50S and 20% of the <50S poly(A)hnRNA failed to bind during poly(U)–Sepharose chromatography. Therefore, we tested the efficiency of oligo(dT)-cellulose in the recovery of poly(A)hnRNA. In Fig. 5A, the binding and elution of poly(A)hnRNA are shown. We found that 20–22% of the total nuclear RNA from mouse brain is polyadenylated. Since partial degradation might make "buried" poly(A) available for hybridization, we treated the unbound or 0.5 M NaCl fraction in Fig. 5A with 0.2 N NaOH for 3 sec in order to nick the RNA (5). Sedimentation in a DMSO–sucrose gradient showed that the mass average of the RNA was reduced by about 40% after this treatment. Figure 5B shows that after brief treatment with alkali no additional binding was observed. [^{3}H]poly(U) also failed to hybridize in measurable quantities to the 0.5 M NaCl fraction of Fig. 5B. Molloy *et al.* (20) reported increased binding of hnRNA to poly(U)–Sepharose after partial alkali degradation.

In another test, 500 μg of unbound nuclear RNA (0.5 M NaCl fraction, Fig. 5A) was treated with RNAase under conditions in which poly(A) tracts remain while the remaining RNA is digested (2 μg/ml RNAase A, 5 units/ml T$_1$RNAase, 37°C, 30 min in buffered 0.5 M NaCl, pH 7.5) (21). Following this treatment, RNAase was removed with pronase and extraction with phenol–chloroform. The sample was then subjected to oligo(dT)–cellulose chromatography to concentrate the poly(A). The poly(A) was passed through G-50 Sephadex (fine) to remove any oligo(A) and the excluded fraction was reacted with [^{3}H]poly(U) to determine the quantity of poly(A) present. We determined by this assay that 0.31 μg of poly(A) was present in 500 μg of unbound RNA. From the average poly(A) content of poly(A)hnRNA from mouse brain of 3.1% (Bantle and Hahn, in preparation), we calculated that only about 34 μg of poly(A)

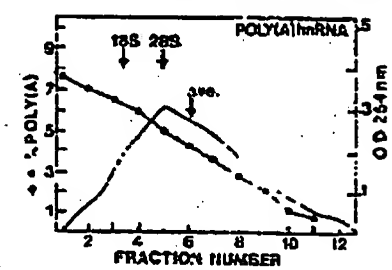

FIG. 6. Poly(A) content in relation to sedimentation rate of poly(A)hnRNA. Poly(A) hnRNA was sedimented in a DMSO–sucrose gradient as in Fig. 2. Fractions were collected. RNA was rebound to oligo(dT)–cellulose to remove DMSO, and optical density at 260 nm measured. The poly(A) content was then determined by hybridization with ^{3}H-labeled poly(U). Optical density scan which is shown was made during fractionation of the gradient.

poly(A) binding capacity, is the purity of the cellulose. T3I is prepared using Whatman CF-11 cellulose washed by the method of Alberts and Herrick (13). Nonspecific binding is probably due to contaminants in the cellulose, especially lignins (14). Washing according to the Alberts and Herrick method apparently reduces these contaminants. Nonspecific binding "sites" have limited capacity and once they are saturated additional binding does not occur. The limited capacity of these sites is demonstrated in Table 2. Notice that 1 g of T3I binds only 0.5–0.7 μg of *E. coli* RNA.

Long-term labeled total *E. coli* RNA apparently contains a small amount (0.004%) of rebindable poly(A)RNA (15). *E. coli* RNA used in these experiments was degraded by the small amount of RNAase present in the DNAase and thus less than 0.004% binding of total RNA would be expected. Hence, virtually all of the binding shown in Table 2 is nonspecific.

We found that it is possible to essentially eliminate nonspecific binding (binding not due to A-T hybridization). Shown in Table 3 is the comparison of binding of nonpolyadenylated nucleic acids to oligo(dT)–cellulose with and without preemption with *E. coli* RNA. When small amounts of labeled double and single strand DNA or poly(A)$^-$RNA are applied to oligo(dT)–cellulose (T3), some binding occurs. If the column is first preempted with *E. coli* RNA, nonspecific binding is essentially eliminated.

Tight binding is a problem encountered when oligo(dT)–cellulose is used to assay for poly(A)RNA present in samples containing only a few micrograms of isotopically labeled RNA. Here, poly(A)RNA is bound by some mechanism other than A-T hybridization and hydrolysis is required for the removal of the bound RNA shown in Table 4. When small amounts (1–2 μg) of poly(A)RNA are applied to 0.5 g oligo(dT)–cellulose columns, nearly 40% of the RNA is not eluted under conditions which dissociate A-T duplexes (e.g., 0.01 M tris–HCl, H_2O, and 90% formamide). Elution of the tightly bound RNA requires extensive hydrolysis (overnight treatment with 0.1 N NaOH). As is the case for the H_2O-elutable nonspecific binding, tight binding is of low capacity and can also be preempted with *E. coli* RNA. Table 4 shows that preemption essentially eliminates tight binding of poly(A)RNA.

TABLE 3

PREVENTION OF NONSPECIFIC LOW CAPACITY BINDING[a]

Nucleic Acid	Bound preempted column (%)	Bound untreated column (%)
Sheared single strand DNA	0.1	3.3
Sheared double strand DNA	0.3	8
4S *E. coli* RNA	0.2	2.5

[a] In each case 1–2 μg of ^{3}H-labeled nucleic acid (7000–25000 cpm) was applied under standard conditions and the radioactivity in the bound fraction measured. Columns were preempted by a single passage of 100 μg of *E. coli* RNA and washed to background optical density with 0.5 M NaCl prior to addition of the test samples.

TABLE 4

TIGHT BINDING OF POLY(A)RNA[a]

Elution conditions	Preempted column (% eluted)	Untreated column (% eluted)
0.01 M tris–HCl	97.3	57.6
H_2O, 50°C	1.0	2.6
90% formamide, 50°C	0.2	0.5
0.1 N NaOH	1.5	39.3

[a] ^{3}H-labeled poly(A)mRNA (1 μg, 5400 cpm) was applied to 0.5 g oligo(dT)–cellulose. *E. coli* RNA was used to preempt nonspecific binding. After washing to background level of radioactivity with 0.5 M NaCl (about 40% of the poly(A)RNA did not bind), columns were eluted sequentially as indicated.

From these observations on nonspecific binding "sites," it is apparent that when oligo(dT)–cellulose is used to analyze *small amounts* of labeled RNA, preemption of these sites is essential. However, since these sites have a very low capacity, preemption is not critical when oligo(dT)–cellulose is used preparatively.

As shown in Table 2, the extent to which nonspecific binding occurs is related to the purity of the cellulose used. We therefore recommend that cellulose used in preparing oligo(dT)–cellulose be washed according to the procedure of Alberts and Herrick (13).

Elimination of Indirect Binding of rRNA

Initially, we found that poly(A)mRNA prepared by oligo(dT)–cellulose chromatography from total polysomal RNA did not rebind efficiently. Instead, variable rebinding was obtained ranging from 50–85%. Similar results have been obtained by others (16) (Greenberg and Perry, personal communication). We have found that failure to achieve 100% rebinding is due to an impure preparation of poly(A)mRNA. The major contaminant is rRNA which aggregates with poly(A)RNA and hence binds indirectly to oligo(dT)–cellulose. When the poly(A)mRNA is reapplied to oligo(dT)–cellulose, some of the aggregates of poly(A)RNA and rRNA dissociate and the released rRNA is mistaken for poly(A)RNA which did not rebind. Aggregation of rRNA with DNA, mRNA, and poly(A)RNA has been previously observed respectively by Opara-Kubinska *et al.* (17); Hayes *et al.* (18); Suzuki *et al.* (19).

could be present in the 1670 μg of unbound nuclear RNA shown in Fig. 4. Hence, about 93% (470 of 504 μg) of the total poly(A) RNA estimated to be present by these combined assays was recovered.

In Fig. 6, the sedimentation and poly(A) content of poly(A)hnRNA is shown. The poly(A) content decreased with increasing sedimentation rate. This was as expected, since the poly(A) tracts of mouse brain hnRNA have a fairly narrow size range (160–250; Bantle and Hahn, in preparation), and thus higher molecular weight species would contain proportionally less poly(A). Finally, repeated binding of poly(A)hnRNA to oligo(dT)–cellulose does not alter the sedimentation pattern in denaturing gradients. This indicates that nicking of poly(A)RNA is not a serious problem in oligo(dT)–cellulose chromatography.

DISCUSSION

Our evaluation of oligo(dT)–cellulose shows that direct nonspecific binding occurs. Nonspecific binding can lead to errors when oligo(dT)–cellulose is used to assay for poly(A)RNA present in microgram or submicrogram samples of RNA as given in Tables 2 and 3. Falsely high values for poly(A)RNA content can result due to the presence of nonpolyadenylated RNA which binds either directly, or indirectly, as an aggregate with poly(A)RNA. Low estimates of poly(A)RNA can also occur due to tight binding in which poly(A) RNA is not eluted under conditions which dissociate A-T duplexes. Fortunately, these nonspecific binding sites have a very low capacity. They can be readily preempted by treatment of the column with bacterial RNA, as shown in Tables 3 and 4. Singer and Penman (3) used tRNA from yeast to reduce nonspecific binding. Preemption of nonspecific binding permits assays to be based solely on A-T hybridization.

When oligo(dT)–cellulose columns are used preparatively, as, for example, to isolate poly(A)mRNA from bulk polysomal RNA, the nonspecific binding sites present no serious problems because of their low capacity. However, preparative and analytical procedures both require disaggregation steps in order to prevent the indirect binding of nonpolyadenylated RNA. In the preparation of poly(A)mRNA we have found that disaggregation is best performed after most of the rRNA is eliminated by the initial passage through oligo(dT)–cellulose. Once poly(A)mRNA has been freed of rRNA, it may be repeatedly applied to oligo(dT)–cellulose with the result that 97–98% rebinding and recovery is obtained. This indicates that nicking of poly(A)mRNA is not a serious problem in oligo(dT)–cellulose chromatography.

As shown in Fig. 4, efficient recovery of poly(A)hnRNA from nuclear RNA is obtained by oligo(dT)–cellulose chromatography provided a disaggregation step is employed prior to passage through the column. Using this procedure, we find that 19–22% of the nuclear RNA from mouse brain is polyadenylated. Only 9–11% of the nuclear RNA was recovered as poly(A)RNA when the DMSO–heat disaggregation was not performed prior to chromatography. The loss in this latter case may be random because saturation hybridization experiments (Bantle and Hahn, unpublished data) show similar diversity values for both the high and low yield preparations of poly(A)hnRNA. Rebinding of poly(A)hnRNA approaches 100%, providing disaggregation procedures are used. For example, aliquots of RNA taken from various fractions of the gradient displayed in Fig. 5 rebound 95%.

In summary, we conclude that the analytical precision of oligo(dT)–cellulose chromatography is excellent provided nonspecific binding is prevented and that efficient recovery of poly(A)mRNA and poly(A)hnRNA is possible if procedures which eliminate aggregation are employed.

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